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SEASONAL VARIATION IN AGGLUTINATION OF *PLASMODIUM FALCIPARUM*-INFECTED ERYTHROCYTES

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Abstract. Agglutination and rosette formation are in vitro characteristics of *Plasmodium falciparum*-infected erythrocytes, which have been associated with host protective immune responses and also with parasite virulence. The present study was carried out in an area of seasonal and unstable malaria transmission in eastern Sudan. Plasma samples were obtained before, during, and after the transmission season from a volunteer cohort of 64 individuals seven years of age and older. These plasmas were assayed for their ability to agglutinate cultured parasitized erythrocytes originally obtained from acute malaria infection samples taken from five of the cohort members. Our data show that the capacity of donor plasma samples to agglutinate parasitized cells depended largely on the time of sampling relative to the transmission season, at least within this epidemiologic setting. Thus, although less than half of the pretransmission season samples could agglutinate any of the five lines of cultured parasites, all post-transmission season samples could agglutinate at least one of the parasite lines, with 74% agglutinating two or more lines. This increase in the agglutination capacity of individual plasma samples after the transmission season occurred essentially regardless of whether an individual had experienced a clinical malaria attack during the transmission season. The study thus confirms the acquisition of agglutinating antibodies following episodes of clinical malaria, but also demonstrates that such acquisition can take place in the absence of disease, presumably as a consequence of subclinical infection. This is the first demonstration of marked seasonal fluctuations in the capacity of individuals' sera to agglutinate parasitized red blood cells. Possible explanations for this effect include a decrease in the levels of agglutinating antibodies between seasons, or shifts in the antigens being recognized by such antibodies from one transmission season to the next. Finally, we showed the existence of marked seasonal fluctuation in the levels of agglutinating antibodies, either because levels of such antibodies are not sustained between seasons or because the antigens recognized change from one season to the next.

Erythrocytes infected by *Plasmodium falciparum* express highly variable parasite-derived neoantigens at the red blood cell surface membrane during the later stages of their development.^{1–3} Predominant among these antigens is PfEMP-1, an extremely polymorphic molecule encoded by the recently identified *var* gene family.^{4–6} Antibodies responsible for agglutination (cross-linking) of *P. falciparum*-infected erythrocytes have been shown to specifically recognize variable antigens on the surface membrane of infected erythrocytes,^{7,8} and acquisition of protective immunity has been proposed to reflect the cumulative recognition of such antigens.⁹ In line with this hypothesis, the ability of sera to agglutinate parasitized erythrocytes has been shown to correlate with relative protection of the serum donor from clinical disease in a study from The Gambia.¹⁰

The present study was designed to investigate plasma-induced parasite agglutination in a region of seasonal and unstable malaria transmission. We examined the relationship between the agglutination properties of the plasma samples, the time the samples were collected relative to the malaria season, and whether the plasma donors had clinical malaria episodes during the malaria season.

MATERIALS AND METHODS

Study area. The study was conducted in Daraweesh, a village of 120 thatched mud huts situated 14 km south of the town of Gedaref, Sudan, 450 km southeast of the capital of Khartoum. Bed nets and chemoprophylaxis are not used

in the village. The area is mesoendemic for *P. falciparum* malaria, with seasonal and unstable transmission, usually peaking in October–November, following the annual rainy season.^{11,12} Little, if any, transmission occurs from January through July. The predominant malaria species is *P. falciparum* (98%), with *P. vivax* and *P. malariae* occasionally seen.

Donors. A cohort of 64 individuals (age range = 7–32 years) volunteered to participate in the study. Participating individuals were requested to present to a health-team present in the village on a daily basis when not feeling well.¹¹ No carriers of the sickle-cell hemoglobin (HbS) allele were included in the study. The study received ethical clearance from The Ethical Committee of the University of Khartoum.

Plasma. Blood samples (15–20 ml) were collected by venipuncture into heparinized vacutainers (Becton Dickinson, Rutherford, NJ). A total of 144 blood samples were collected after informed consent was obtained. Fifty-four of the samples were collected at the beginning of the malaria season in September, when all donors were without clinical or microscopic evidence of malaria infection. An additional sample was collected from 28 of 31 cohort donors who experienced uncomplicated, microscopically confirmed, *P. falciparum* malaria during the season. Finally, a total of 62 samples were collected at the end of the season in December–January, when all donors were again without evidence of malaria. Of these latter samples, 29 were obtained from donors who had had at least one clinical malaria episode during the malaria season, while the remaining were from donors without such episodes.

Following centrifugation of the blood samples, plasma was collected and kept frozen at -20°C until use. In addition, aliquots of the erythrocyte pellet were stored frozen in liquid nitrogen or at -80°C for subsequent parasite polymerase chain reaction (PCR) analysis.

Parasites. From five donors with clinical *P. falciparum* malaria infections, pelleted parasitized erythrocytes were collected from the blood sample, washed three times in RPMI 1640 medium (Gibco, Paisley, United Kingdom), resuspended in glycerol cryopreservation medium, and transferred to liquid nitrogen as previously described,¹³ where they were stored until brought into *in vitro* culture. The parasite donors were a 10-year-old male (2I4), a 12-year-old female (X7), a 17-year-old female (2L5), a 20-year-old female (2A3), and a 32-year-old male (2I1).

Parasite isolates from the above donors were cultured as previously described.¹⁴ For the first multiplication cycle, the parasites were kept in autologous erythrocytes, while all the isolates were subsequently maintained in blood-group O⁺ erythrocytes from a single donor. Medium was changed, and parasite growth was monitored six times a week.

Microagglutination assay. The ability of plasma samples to induce antibody-mediated agglutination of parasitized erythrocytes was measured by modifications of previously published protocols.^{15,16} Parasites multiplied *in vitro* until parasitemias of at least 1% were reached. Erythrocytes were then washed in phosphate-buffered saline (PBS) and the haematocrit adjusted to 20%. Fifty-microliter aliquots of erythrocyte pellet, 25 μl of PBS containing 40 $\mu\text{g}/\text{ml}$ of ethidium bromide, and 5 μl of test plasma were dispensed into 96-well microtiter plates (Nunc, Rødovre, Denmark). The plates were then sealed and incubated on a shaker (10 rpm) at 37°C for 1 hr. Subsequently, 1- μl samples were mounted on multipoint slides under coverslips and examined microscopically under visual and UV light. The ability to agglutinate and size of the largest agglutinates were recorded.

Parasite PCR. Parasite DNA was prepared from erythrocyte pellets essentially as described.¹⁷ Briefly, 20 μl of erythrocyte pellet was added to 500 μl of ice-cold 5 mM sodium phosphate (pH 8), vortexed, and centrifuged for 10 min. This washing procedure was repeated four times. Finally, pellets were resuspended in 50 μl of sterile water, and boiled for 10 min before storage at -70°C until use as template DNA in the PCR.

The PCR analysis was carried out essentially as previously described.¹⁷ Genotyping of the parasite DNA samples was carried out using the *P. falciparum* merozoite surface protein-1 (MSP-1) gene block 2-specific primer pairs,¹⁸ the *P. falciparum* MSP-2 gene IC1 and FC27 primer pairs,¹⁹ and primer pairs flanking the variable repeats of the glutamate-rich protein (GLURP) gene.²⁰

Statistical analysis. Differences in proportions were analyzed by the chi-square test. Analysis of factors affecting the agglutination capacity of plasmas was done by multiple linear regression. SigmaStat software (Jandel Scientific, San Rafael, CA) was used for statistical analysis.

RESULTS

Agglutination properties of plasma samples. All 144 plasma samples were tested for their ability to induce agglu-

tinate each of the five parasite isolates (Figure 1). Overall, 35% (19 of 54) of the pre-season (September) samples induced agglutination in one isolate and 11% (6 of 54) did so in two isolates. The remaining 29 September samples did not agglutinate any isolates. On average, the pre-season samples agglutinated 0.6 ± 0.6 isolates per sample (mean \pm SD). The proportions of plasmas able to agglutinate 0, 1, or 2 parasite isolates were not statistically different between donors who subsequently came down with clinical malaria and those who did not ($P = 0.09$, by chi-square test). However, the power of the test to detect differences was relatively low due to the limited sample size.

Of the 28 acute plasmas, 39% (11) were unable to agglutinate any isolate, while 36% (10), 21% (6), and 4% (1) agglutinated 1, 2, and 3 isolates, respectively. Average agglutination was 0.9 ± 0.9 isolate/plasma sample. The proportions of acute plasmas able to agglutinate one or more parasite isolates were not statistically different from the pre-season samples from the same individuals ($P = 0.27$, by chi-square test).

At the end of the malaria season, the situation had changed dramatically because all 62 plasma samples now agglutinated at least one parasite isolate. Twenty-six percent (16) of the samples agglutinated one isolate, while 44% (27), 26% (16), 3% (2), and 2% (1) were able to agglutinate 2, 3, 4, and all 5 isolates, respectively, yielding an average of 2.1 ± 0.9 isolates/plasma sample. However, as for the pre-season samples, the difference in the proportions of the post-season plasmas to agglutinate different numbers of isolates was not statistically different ($P = 0.08$, by chi-square test) between individuals with and without a previous disease episode, although again the power of the test to detect genuine differences was suboptimal.

In contrast, the proportion of plasmas able to agglutinate different numbers of isolates after the transmission season was significantly different from that before the season. This was true regardless of whether all samples were analyzed together, or separated into those with (Figure 2) and without (Figure 3) clinical episodes during the season ($P < 0.001$ in all cases, by chi-square test).

Thus, plasma agglutination capacity increased markedly over the transmission season, although agglutination capacity did not appear to play a significant role in protection against clinical malaria, at least in the short term. This conclusion is supported by analysis of all the agglutination data by multiple linear regression. This analysis used time of sampling, whether the donor had a clinical episode, and donor age as independent variables to predict the number of parasite isolates agglutinated by a given sample. Only the first of these variables (i.e., sampling before or after the transmission season) significantly affected this number ($P < 0.001$).

A plasma pool from Danish donors who had not been exposed to malaria did not induce agglutination in any of the isolates.

Agglutination capacity in relation to clinical history. We examined whether there were differences in the clinical histories between the individuals from whom pre-season plasma agglutination data were available with respect to the agglutination capacity of their pre-season samples. This was done to test whether individuals who were known to have

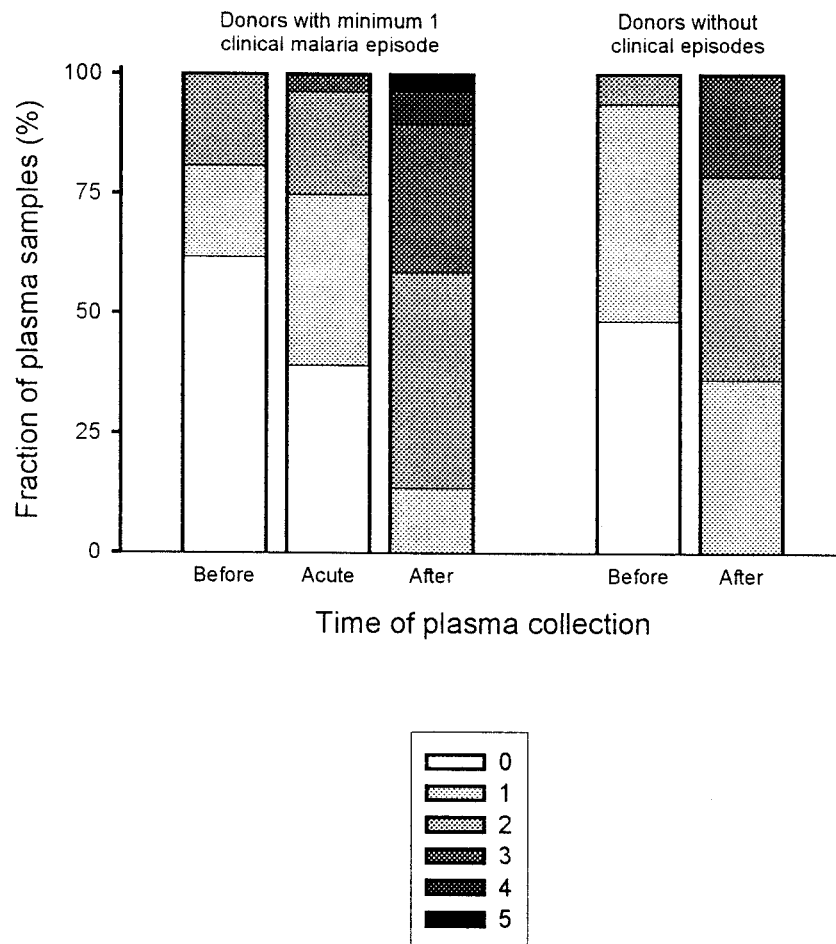


FIGURE 1. Proportions of plasma samples able to agglutinate parasite isolates in relation to time of sampling. Samples were collected before the malaria season, during acute clinical malaria attacks, and after the malaria season from individuals who did (**left**) or did not (**right**) experience a clinical malaria attack during the malaria season. The proportions of samples able to agglutinate 0, 1, 2, 3, 4, and 5 isolates are indicated by no, light, medium, dark, very dark, and black shading, respectively.

had a clinical malaria episode in the previous 12 months (31 of 54) were more likely to be able to agglutinate parasitized red blood cells than those who had not had malaria in the past year (23 of 54). There were no statistically significant differences in this respect between those whose pre-season samples were able to agglutinate 0, 1, or 2 parasite isolates, respectively. Thus, the recent malaria clinical history of the individuals had no apparent impact on the ability of the pre-season samples to induce agglutination. This suggests either that at least some species of agglutinating antibodies are of very short duration, or that there are marked differences between the target antigens of agglutinating antibodies present in any given season and the next.

Parasite isolate characterization by agglutination. The characteristics of the five parasite isolates used in the agglutination assays are summarized in Table 1. Two isolates (2I4 and X7) were readily agglutinated by a large proportion of the plasmas, particularly by post-season plasmas, while two (2A3 and 2I1) were only infrequently agglutinated, and not by pre-season and acute plasmas. No isolates were agglutinated by the three autologous pre-season plasmas available, and only one of five were agglutinated by the autologous acute plasma sample (Figure 2). In contrast, four of five of

the isolates were agglutinated by post-season autologous plasma (Figure 2). All isolates were agglutinated least by pre-season plasmas, and most by post-season plasmas, with acute plasmas being intermediate. This correlation was statistically significant in all cases ($P = 0.02$ – 0.001 , by chi-square test).

Strikingly, the parasite isolates were agglutinated equally well by plasmas from donors with and without clinical malaria episodes, both pre-seasonally ($P > 0.87$, by chi-square test; only isolates 2I4 and X7 were tested) and post-seasonally ($P = 0.1$ – 0.8 in all cases, by chi-square test).

The age of the isolate donor correlated inversely with the number of plasmas able to agglutinate the isolate, although not reaching conventional statistical significance ($P = 0.10$, $r = -0.81$).

Parasite detection and genotyping by PCR. Three of the isolates (X7, 2I4, and 2I1) amplified single alleles in each of the MSP-1, MSP-2, and GLURP genotyping assays. Given that the blood stream forms of *P. falciparum* are haploid, this result is consistent with each of these isolates being a single clone. The remaining two isolates (2A3 and 2L5) amplified more than one allele in these genotyping assays, and were deduced to contain a minimum of two and three clones,

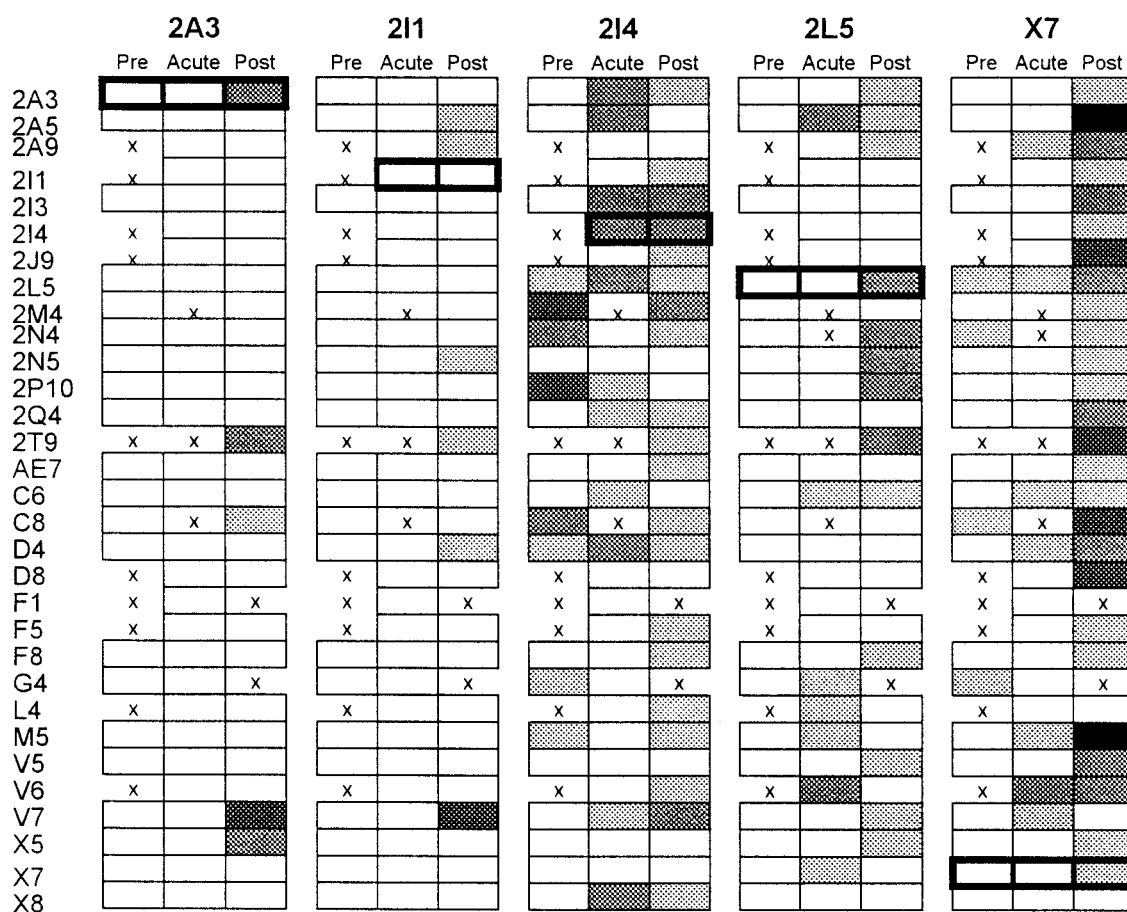


FIGURE 2. Agglutination of five different parasite isolates by plasma samples obtained before and after the malaria season, and during acute disease, from 31 individuals experiencing clinical malaria episodes during the season. Degree of agglutination is represented on a five-level semiquantitative scale.⁷ Autologous isolate/plasma combinations are emphasized by the heavy frames. X = no sample available.

respectively. None of the isolates were identical for all the markers examined.

Twenty-four percent (13 of 54) of the blood samples collected in September had a *P. falciparum* parasitemia that was detectable by nested PCR analysis,¹² yet was not detected by conventional light microscopy of Giemsa-stained smears. Of these, six of 21 were from individuals who subsequently came down with clinical malaria in that season, while seven of 33 were from individuals who did not. Thus, the proportions were similar in these two groups of donors ($P = 0.77$, by chi-square test), indicating that the presence of PCR-detectable *P. falciparum* infection prior to the transmission season does not predict subsequent clinical disease. All 26 acute samples examined tested positive by PCR (as by microscopy), while 19% (12 of 62) of the postseason samples were positive by PCR (but negative by microscopy). Of these latter samples, six of 29 were from donors who had had a malaria attack that season, and six of 33 were from donors who had not had a malaria attack that season ($P = 0.94$, by chi-square test).

DISCUSSION

Agglutinating antibody responses targeted at parasite-derived erythrocyte surface antigens during and following clin-

ical malaria episodes are potentially important protective immune responses that may control parasite infections.²¹ These parasite-derived erythrocyte surface antigens are currently thought to mediate mature asexual stage sequestration and clonal antigenic variation.²² It is likely that all of these properties of *P. falciparum* infections are mediated by the variable erythrocyte surface antigens, now known as PfEMP-1,²³ encoded by the *var* genes.⁴⁻⁶ It has also been shown that the immunologic capacity to recognize PfEMP-1 serotypes correlates with age in humans living in areas of high malaria endemicity. Thus, children are generally able to recognize only a limited number of serotypes, whereas clinically immune adults have antibodies capable of recognizing many more antigenic types.^{9,10}

This study was conducted in an area mesoendemic for malaria, where malaria morbidity is restricted to a three-month period at the end of the annual rainy season. Patent parasitemia outside this period is rarely seen. The entomologic inoculation rate in the area has been estimated to be approximately 0.6 infective bites per person per year (Hamid AA, unpublished data). However, intensity of transmission is subject to large fluctuations between years because the area is prone to droughts when parasite prevalence may decrease to very low values (Elhassan IM, unpublished data). It is possible that under these conditions even older individ-

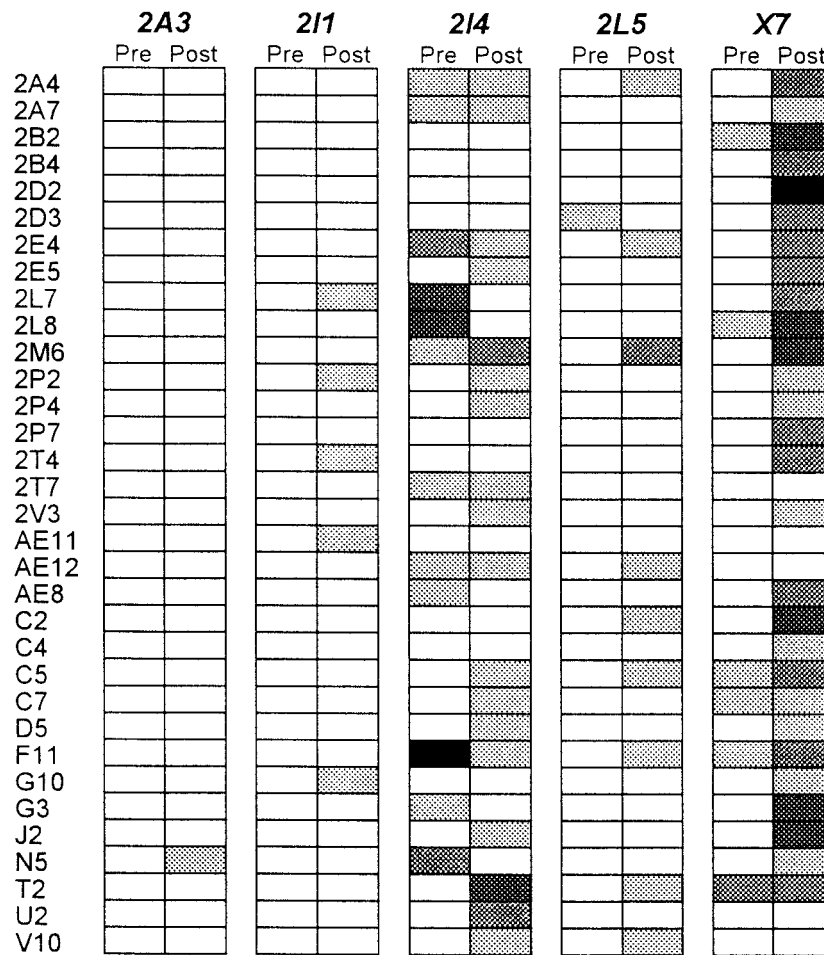


FIGURE 3. Agglutination of five different parasite isolates by plasma samples obtained before and after the malaria season from 33 individuals without clinical malaria episodes during the season. See Figure 2 for additional information.

uals do not reach a level of immunity where they possess agglutinins able to recognize most or all parasite isolates, in contrast to what is seen in areas of high endemicity.^{7, 9, 10, 24, 25} Consistent with this reasoning, we found that more than half of the plasma samples collected at the beginning of the malaria season were unable to agglutinate any of the parasite isolates used, and only about 10% agglutinated more than one isolate. Similar low figures were found when examining plasmas from patients with acute malaria, in line with previous data.⁷ In contrast, all plasma samples collected at the end of the season (at least 30 days after the last clinical episode seen in the village) agglutinated at least one of the

five parasite isolates collected in the village that season. More than 80% of these plasma samples agglutinated two or more parasite isolates. In a study from The Gambia, Marsh and others¹⁰ found levels of agglutinating antibodies to be the only *in vitro* parameter correlating with protection. However, when comparing agglutination in the postseason samples from donors with and without preceding clinical episodes in the present study, also conducted in an area of seasonal, but much lower, malaria transmission, we were unable to detect any difference in plasma agglutination between these groups.

Significantly, our data demonstrate for the first time that

TABLE 1
Agglutination characteristics of *Plasmodium falciparum* isolates*

| Isolate | Donor age (years) | Preseason | | Clinical episode (n = 28) | Postseason | |
|---------|-------------------|-------------|--------------|---------------------------|-------------|--------------|
| | | CE (n = 21) | NCE (n = 33) | | CE (n = 29) | NCE (n = 33) |
| 2I4 | 10 | 0.38 (8) | 0.36 (12) | 0.39 (11) | 0.69 (20) | 0.55 (18) |
| X7 | 12 | 0.19 (4) | 0.18 (6) | 0.25 (7) | 0.90 (26) | 0.85 (28) |
| 2L5 | 17 | 0 | 0.03 (1) | 0.25 (7) | 0.45 (13) | 0.27 (9) |
| 2A3 | 20 | 0 | 0 | 0 | 0.17 (5) | 0.03 (1) |
| 2I1 | 32 | 0 | 0 | 0 | 0.21 (6) | 0.15 (5) |
| Overall | — | 0.11 | 0.12 | 0.18 | 0.48 | 0.37 |

* CE = clinical episode during the season; NCE = no clinical episode during the season. Values are the fraction of all plasmas (N) agglutinating an isolate.

acquisition of agglutinating antibodies can occur in the absence of clinical disease. This implies that seroconversion to recognition of the considered target of agglutinating antibodies, i.e., the antigenically variable PfEMP-1 molecule, is occurring during chronic asymptomatic infections. That a marked proportion of infections in this area run a subclinical course is corroborated by our finding of PCR-detectable infections at the end of the season in approximately 20% of the donors without clinical episodes, and by our previous serologic and PCR data from the same area.^{11,12}

It has been speculated that acquisition of clinical immunity to malaria is associated with accumulation of a sufficient diversity of strain-specific agglutinating antibodies to enable recognition of a large proportion of parasite isolates (pan-agglutination).²⁶ However, we did not find any association between age and agglutinins in the present study, and perhaps the rate at which individuals are exposed to different parasite strains in our study area is simply too low to make such an association apparent. On average, irrespective of age, individuals in Daraweesh have an attack of clinical malaria once every 2–3 years.

An alternative explanation is that the variability of parasite strains transmitted within the small community we have studied may be lower than those in previous studies, at least as far as antigens mediating agglutination are concerned. We do not favor this explanation since the parasite population in our study area has been demonstrated to be genetically diverse.^{27–30}

We believe that this study constitutes an important addition to the surveys previously carried out^{7,9,24,25} in demonstrating marked seasonal variation and lack of age structuring in agglutination capacity and seroconversion in the absence of clinical disease in an area of low and unstable malaria transmission.

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